

Astrotactin provides a receptor system for CNS neuronal migration

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Summary

CNS neuronal migration is a specialized form of cell motility that sets forth the laminar structure of cortical regions of brain. To define the neuronal receptor systems in glial-guided neuronal migration, an *in vitro* assay was developed for mouse cerebellar granule neurons, which provides simultaneous tracking of hundreds of migrating neurons. Three general classes of receptor systems were analyzed, the neuron–glial adhesion ligand astrotactin, the neural cell adhesion molecules of the IgG superfamily, N-CAM, L1 and TAG-1, and the β_1 subunit of the integrin family. In the absence of immune activities, migrating cerebellar granule neurons had an average *in vitro* migration rate of $12 \mu\text{m h}^{-1}$, with individual neurons exhibiting migration rates over a range between 0 to $70 \mu\text{m h}^{-1}$. The addition of anti-astrotactin antibodies (or Fabs) significantly reduced the mean rate of neuronal migration by sixty-one percent, resulting in eighty percent of the neurons having migration rates below $8 \mu\text{m h}^{-1}$. By contrast, blocking antibodies (or Fabs) against L1,

N-CAM, TAG-1 or β_1 integrin, individually or in combination, did not reduce the rate of neuronal migration.

By video-enhanced contrast differential interference contrast microscopy the effects of anti-astrotactin antibodies were seen to be rapid. Within fifteen minutes of antibody application, streaming of cytoplasmic organelles into the leading process arrested, the nucleus shifted from a caudal to a central position, and the extension of filopodia and lamellopodia along the leading process ceased. Correlated video and electron microscopy suggested that the mechanism of arrest by anti-astrotactin antibodies involved the failure to form new adhesion sites along the leading process and the disorganization of cytoskeletal components. These results suggest astrotactin acts as a neuronal receptor for granule neuron migration along astroglial fibers.

Key words: cerebellum, neuronal migration, astroglia, neuron–glia binding.

Introduction

Mammalian embryogenesis is characterized by extensive cell movements, ranging from the restricted migration of germ cells to colonize the primordial ridge to the wide-ranging migrations of neural crest cells to establish the peripheral nervous system. Whereas cellular movements outside the CNS appear to be guided by components of the extracellular matrix (Hynes, 1990), neuronal migrations in cortical regions of brain are guided by a system of radial glial fibers (Rakic, 1972; Rakic, 1990; Misson *et al.* 1991). The close apposition of migrating neurons to glial fibers (Rakic *et al.* 1974; Edmondson and Hatten, 1987; Gregory *et al.* 1988) has suggested that cell surface receptor systems provide an adhesion mechanism for neuronal locomotion along glial fibers (Hatten, 1990).

The cerebellum has provided an opportune model for studies on CNS migration because the granule neuron, one of the most abundant neurons in brain, undergoes

glial-guided migration from the external granule cell layer along the Bergmann glial fibers (Ramon y Cajal, 1911; Rakic, 1971). To analyze glial-guided neuronal migration, we have developed an *in vitro* model system and examined the migration of living granule cells along single glial fibers. Video-enhanced contrast differential interference contrast microscopy (AVEC-DIC) reveals that the migrating neuron assumes an elongated profile on the glial arm, positioning its nucleus in the anterior portion of the cell body and tapering its rostral portion into a motile leading process which extends along the glial fiber (Edmondson and Hatten, 1987). The motion of the neuronal cell body along the glial guide is saltatory, with cells moving in 4 to 8 min cycles (Edmondson and Hatten, 1987). During migration, a specialized neuron–glia junction forms along the length of the neural soma, termed an interstitial junction (Gregory *et al.* 1988), and intracellular vesicles stream from the cytoplasm into the leading process. When cells stop migrating, or pause along the glial fiber, the

neurons adopt a rounded morphology, maintaining their attachment to the glial fiber through puncta adherentia (Gregory *et al.* 1988). Studies on hippocampal neurons and on mosaic cultures of hippocampal and cerebellar cells (Gasser and Hatten, 1990*a,b*) provide evidence that cytology, neuron–glia apposition and dynamics of movement of cerebellar granule neurons along glial fibers are prototypic of neurons in other brain regions.

The motility of migratory CNS neurons differs from that of neural crest cells and growth cones in several respects. First, whereas the latter spread onto the substratum and move forward *via* the formation of focal adhesion sites at their leading edge, the leading process of the migrating CNS neuron has a tapered configuration with short filopodia and lamellopodia extending along the shaft of the leading process to envelope the glial fiber. Second, although the lamellopodial extensions of the leading edge of neural crest cells and of neural growth cones act to promote forward motion, the motion of the leading process is not correlated with the forward movement of the neural soma (Edmondson and Hatten, 1987). Third, CNS neuronal migration appears to involve the formation of an interstitial junction in migrating cells, rather than focal adhesions at the growing tip. (Gregory *et al.* 1988). *In vitro* analyses of axonal growth cone locomotion in both vertebrate systems and in *Drosophila* suggest that multiple receptor systems, including $\beta 1$ integrins, the neural cell adhesion molecules of the IgG superfamily, N-CAM and L1, and N-cadherins contribute to growth cone locomotion (Tomaselli *et al.* 1986, 1988; Chang *et al.* 1987; Neugebauer *et al.* 1988; Reichardt *et al.* 1989; Lemmon and Lagenaur, 1989; Elkins *et al.* 1990; Drazba and Lemmon, 1990).

To define neuron–glia adhesion systems in CNS glial-guided neuronal migration, we used *in vitro* assays to identify an immune activity, which we named anti-astrotactin, that blocks neuron–glia interactions *in vitro* (Edmondson *et al.* 1988). Western blot and immunoprecipitation analysis indicate that the blocking antibodies recognize a neuronal glycoprotein with an apparent relative molecular mass of approximately 100×10^3 (Edmondson *et al.* 1988). Anti-astrotactin antibodies block the binding of neuronal membranes to glial cells (Stitt and Hatten, 1990) and the establishment of neuron–glia contacts *in vitro* (Edmondson and Hatten, 1987). In contrast, antibodies against the major families of neuron–neuron ligands, N-CAM, L1, NILE, TAG-1 and N-cadherin, do not block any of these neuron–glia interactions *in vitro* (Edmondson *et al.* 1988; Stitt and Hatten, 1990). To examine the contribution of astrotactin, neural cell adhesion molecules of the IgG superfamily and $\beta 1$ integrin to granule neuron migration along astroglial fibers, we have developed an assay to quantify the migration of large populations of granule cells, and examined the perturbation of neuronal migration by antibodies against cell adhesion molecules. These studies provide evidence that astrotactin functions in neuronal locomotion along the glial fiber *in vitro*.

Materials and methods

Microcultures of migrating neurons

An enriched fraction of cerebellar granule neurons (95%) and astroglia (5%) was purified from C57Bl/6J mice on postnatal days 2–6 (Hatten, 1985), and plated as described (Edmondson and Hatten, 1987) at a final cell density of 8.5×10^5 cells ml^{-1} in microcultures (50 μl) (Hatten and Francois, 1981) or on 12 mm coverslips (100 μl) (Fisher Sci. Co). Prior to the addition of the cells, culture surfaces were pretreated with MatrigelTM (Collaborative Research Inc., Lexington, MA; 1:50–1:100, 45 min at 35.5°C) (Gasser and Hatten, 1990*a,b*). One to four hours after plating, cells were transferred to serum-free medium supplemented with insulin (10 $\mu\text{g ml}^{-1}$), transferrin (100 $\mu\text{g ml}^{-1}$), putrescine (100 $\mu\text{g ml}^{-1}$), progesterone (20 nM), selenium (30 nM), guanosine (200 μM), glucose (6.0 mg ml^{-1}), penicillin–streptomycin (20 units ml^{-1} , GIBCO) glutamine (2 mM), and BSA (10 mg ml^{-1}) (Sigma). For microcultures, a second coverslip was placed over the culture well after shifting the cells to serum-free medium. For cell culture on coverslips, the coverslips were inverted in 35 mm dishes and overlaid with medium 1 h after plating. Both culture preparations were maintained at 35.5°C with 100% humidity and 5% CO_2 . All migration assays were carried out 30–40 h after the cells were plated.

Quantitation of neuronal migration with computer assisted, time-lapse phase-contrast microscopy

Neuronal migration was assayed by randomly selecting five fields per culture, identified by scribing with a false objective. Images of cells within identified fields were obtained at twenty to forty minute intervals with a Hamamatsu Chalnicon video camera, mounted on a Zeiss IM35 microscope with phase-contrast microscopy (Zeiss planapochromat 16 \times objective), and recorded with a Panasonic Memory Disk Recorder. An Image-1 computer system (Universal Imaging) was used to track the movement of neurons along glial fibers in each of the scribed fields and determine the average speed and frequency distribution of glial-guided neuronal migration (Fig. 1A). Ten 50 μl microcultures (50 fields) were analyzed in each experiment, for a total average cell sample of 800 cells.

Antisera

Anti-astrotactin antibodies were purified as described (Stitt and Hatten, 1990) from a polyclonal antiserum, raised in rabbits against P8 cerebellar granule cells. By western blot analysis, purified antibodies recognized a neural antigen with a relative molecular mass of approximately 100×10^3 (Stitt and Hatten, 1990). Polyclonal N-CAM antibodies were the gift of Dr C. Goridis (Marsielles), polyclonal L1 antiserum was provided by Dr C. Lagenaur (Pittsburgh), polyclonal TAG-1 antisera was the gift of Drs J. Dodd and T. Jessell (Columbia), and polyclonal anti-rat GP140 antisera against $\beta 1$ integrin was provided by Dr C. Buck (Wistar). IgG fractions were prepared by affinity purification with a protein A column (Pierce), and Fab fragments were prepared using papain digestion (Pierce Immunopure Fab preparation kit). Concentrated (5.0–10.0 mg ml^{-1}) Fabs or IgGs were added to 2 \times serum-free media yielding a final concentration of IgG or Fab of 0.5–1.0 mg ml^{-1} . Combinations of anti-N-CAM/L1/ $\beta 1$ integrin or anti-N-CAM/L1/TAG-1, were prepared by diluting individual antibodies at concentrations between 0.2 and 0.8 mg ml^{-1} .

Analysis of antibody perturbation of neuronal migration along glial fibers with time-lapse phase-contrast microscopy

Cells were plated in microcultures for 20–30 h *in vitro*, after which IgG fractions or Fab fragments ($0.5\text{--}1.0\text{ mg ml}^{-1}$) were added and neuronal migration was measured for 3 h by time-lapse phase-contrast microscopy as given above (Figs 1 and 2). In a single trial, two cultures were treated with each of the four antibodies (or Fabs), or a combination thereof. Five random fields were analyzed 'blind' per culture. Values for the average speed, frequency and distribution of frequencies of migration in each trial were normalized against control, untreated cultures. For the latter, we averaged the values obtained for control, untreated cells in all of the trials and normalized the speed of untreated cells obtained in a given trial against the values obtained for the total control population. For each immune activity, a minimum of three separate trials were run, yielding an average sample of 300–600 migrating neurons.

Analysis of antibody perturbation of neuronal migration along glial fibers with video-enhanced contrast differential interference contrast microscopy

AVEC-DIC microscopy was performed as described (Edmondson and Hatten, 1987), except that cells were plated on glass coverslips and mounted in a Berg chamber ($100\ \mu\text{l}$ total volume). Prior to the addition of immune activities, randomly selected areas ($n=8$) with cells having migratory profiles were observed using AVEC-DIC microscopy (Allen *et al.* 1981) for 4–6 h. Antisera ($0.5\text{--}1.0\text{ mg ml}^{-1}$ IgG or Fab), or combinations of antibodies, were perfused into the Berg chamber at approximately $100\ \mu\text{l min}^{-1}$ using a LKB Peristaltic pump, and images were recorded at 2–5 min intervals for 3 h, after which the antibodies were removed by perfusion of serum-free media. Images were acquired every five minutes in the case of low power assays, and every two minutes in the case of high power assays. Analysis of each immune activity was done at low power, using a Zeiss Axiovert microscope with Nomarski optics fitted with a Zeiss Plan-Neofluor $20\times/1.6$ objective ($n=6$). In addition, high power AVEC-DIC microscopy assays for anti-astrotactin immune activity, using a Zeiss Plan-Neofluor $100\times/1.6$ oil immersion objective ($n=2$), were also performed (Fig. 3). Images were acquired with a Hamamatsu Chalcon camera and recorded with a Panasonic Memory Disk Recorder. As a control, migration was monitored for 9 h in the absence of immune activities, perfused with fresh media, and further observed with AVEC-DIC microscopy for 5 h. Approximately 50 migrating neurons were examined with AVEC-DIC microscopy for each immune activity assayed.

Correlated video and electron microscopy of migrating neurons in the presence of astrotactin antibodies

To provide positive evidence of migration, prior to processing for EM, we identified migrating cells by AVEC-DIC microscopy as above. For antibody perturbation studies, identified fields were scribed and observed for 3 h after the addition of anti-astrotactin Fabs (1.0 mg ml^{-1}) or N-CAM Fabs (1.0 mg ml^{-1}) at low magnification ($n=6$) with AVEC-DIC microscopy. The cells were then fixed and processed for electron microscopy as described by Gregory *et al.* (1988) (Fig. 4). Electron microscopy was performed with a JEOL 100 Seichon microscope. Approximately 40 migrating cells were analyzed with correlated video and electron microscopy.

Immunolocalization of antibodies against astrotactin, N-CAM, L1, TAG-1, and $\beta 1$ integrin on migrating granule neurons in vitro

After 30–40 h *in vitro* in $50\ \mu\text{l}$ microcultures, living cells were incubated in primary antibodies (1:200–1:500) for thirty minutes. After being washed 3 times in PBS, cells were fixed sequentially in 1.0, 2.0 and 4.0% paraformaldehyde in PBS and then treated with secondary FITC-conjugated, goat anti-rabbit antibodies (Cappel, 1:100, 30 min). After a final wash in PBS, and cells were mounted in anti-photobleaching aqueous mountant (Biomedex). Images were acquired with a Zeiss Axiovert microscope, with epifluorescent illumination, fitted with a computer-controlled, motor-driven focusing system and a MRC series 500 Biorad confocal microscope. Neurons with 'migration profiles' (Edmondson and Hatten, 1987) were optically sectioned and recorded for further processing. Images were transferred to a Gould Dianza IP9400 image processor with a MicroVAX II host computer for serial reconstruction (Fig. 5).

Immunostaining with antibodies against glial filament protein (AbGFP) was performed as described previously (Hatten *et al.* 1984). AbGFP was generously provided by Dr R. K. H. Liem (Columbia).

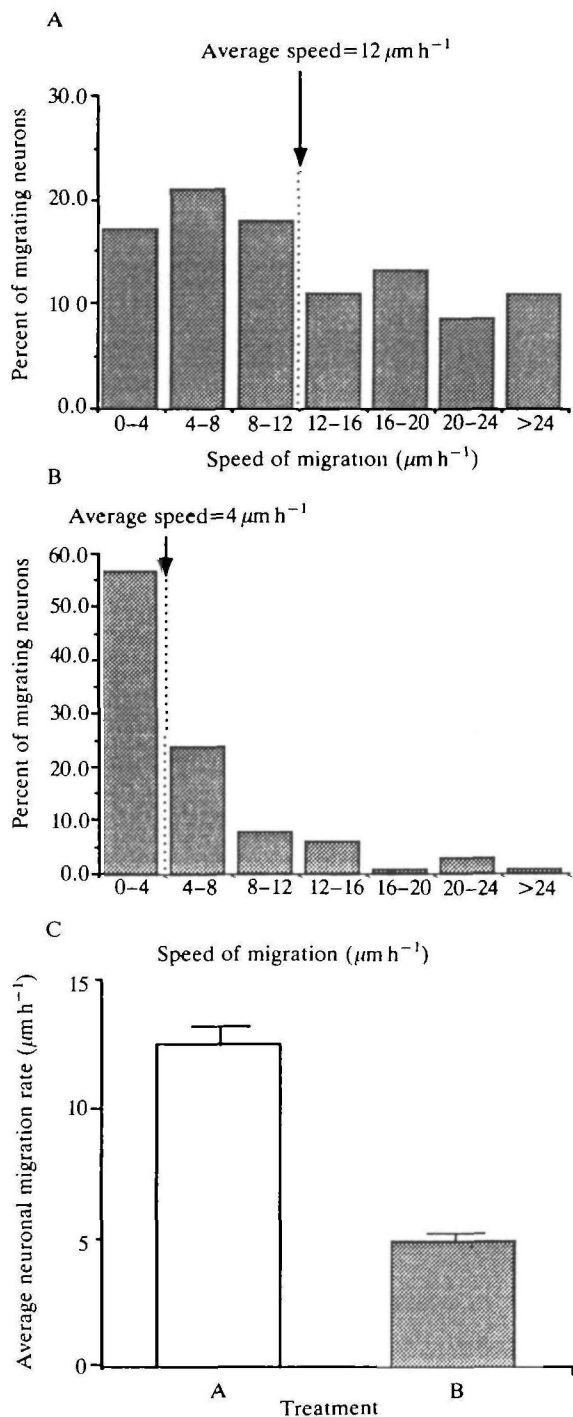
Results

Previous experiments have provided an *in vitro* system for granule cell migration along astroglial fibers (Hatten *et al.* 1984; Edmondson and Hatten, 1987; Gasser and Hatten, 1990b; Hatten, 1990). In the present study, we have quantitated the dynamics of motion of large populations of granule neurons with phase-contrast microscopy and AVEC-DIC microscopy. This system was then used to assay the contribution of astrotactin, the neural cell adhesion molecules of the IgG superfamily, and $\beta 1$ integrin to glial-guided migration.

Measurement of the speed and frequency of migration of granule neurons in vitro

To provide cultures enriched in migrating neurons, we purified cerebellar granule cells at postnatal days 3–5, the period of their migration along Bergmann glia *in vivo*, and co-cultured them with astroglial cells purified from the same tissue. Under these culture conditions, a system of highly elongated ($50\text{--}200\ \mu\text{m}$), AbGF-positive glial fibers developed (results not shown), and granule cells aligned along the glial fibers expressed the profile characteristic of migrating neurons (Hatten *et al.* 1984; Edmondson and Hatten, 1987; Gasser and Hatten, 1990a,b).

To quantify the dynamics of movement of a large population of neurons along the glial fiber system, we simultaneously imaged multiple fields containing a total of approximately 800 cells, at 20 min intervals using phase-contrast microscopy, and measured the average speed and frequency of migration of individual cells with a computer-assisted tracking method (Fig. 1). Over a three hour observation period, approximately eighty percent of the cells moved more than $15\ \mu\text{m}$ (two cell diameters) along a glial fiber (Fig. 1A). The mean rate of migration of the total cell population was $12\ \mu\text{m h}^{-1}$. Within the total population of migrating



neurons, the average speed of individual cells ranged from $0-70 \mu\text{m h}^{-1}$.

To determine whether the neurons moved along the glial fiber system with variable rates or whether differences in the average speed of migration reflected stopping and starting of the cells, we partitioned the migrating neurons into five groups according to their rate of migration, $>24 \mu\text{m h}^{-1}$, $20-24 \mu\text{m h}^{-1}$, $16-20 \mu\text{m h}^{-1}$, $12-16 \mu\text{m h}^{-1}$, $8-12 \mu\text{m h}^{-1}$ and stationary ($0-4 \mu\text{m h}^{-1}$) cells, and examined the frequency distribution of movement of cells with given speeds (Fig. 1A). The frequency distribution of movement suggested that cells

Fig. 1. Anti-astrotactin antibodies reduce both the rate and frequency of granule neuron migration. In A the percent distribution of migratory rates of a population of granule neurons *in vitro* is shown. Cerebellar granule neurons migrate at speeds ranging from 0 to $70 \mu\text{m h}^{-1}$. For this analysis, populations of cells enriched for cerebellar granule neurons were plated on matrigel coated surfaces. Thirty to forty hours after plating, the rate of neuronal migration of large numbers of granule neurons was tracked using phase-contrast microscopy and the speed and frequency of migration of populations of neurons was tabulated. In B the percent distribution of migration rates of a population of granule neurons in the presence of 1.0 mg ml^{-1} anti-astrotactin antibodies is shown. In comparison to the frequency distribution of migratory rates of neurons in control cultures, the majority of neurons are stationary (sixty percent). Of those neurons that continue to migrate, most have speeds of no greater than $8 \mu\text{m h}^{-1}$, significantly below the $12 \mu\text{m h}^{-1}$ average rate observed in control cultures. In C the mean rate of neuronal migration of populations of granule neurons in control (treatment A) and anti-astrotactin antibody (treatment B) conditions is shown. Anti-astrotactin antibodies significantly reduce the mean rate of neuronal migration, from the control level of $12 \mu\text{m h}^{-1}$, to a mean rate of $4-5 \mu\text{m h}^{-1}$.

moving between $0-24 \mu\text{m h}^{-1}$ tracked at all speeds with equal frequency.

Video enhanced contrast differential interference contrast microscopic analysis of granule neuron migration

To examine the dynamics of movement of individual cells in more detail, we imaged the cells every 1-5 min with AVEC-DIC microscopy. Migrating cells expressed a characteristic profile, forming a tight apposition with the astroglial fiber and extending a leading process in the direction of movement. During migration, saltatory contraction and extension of the neural soma along the glial fiber appeared to provide forward motion (Edmondson and Hatten, 1987). Analysis of the rate of movement of individual cells indicated that the mean distance moved per cycle was $5-13 \mu\text{m}$. The total distance moved by individual cells over a 5 h period ranged from $15-210 \mu\text{m}$, with rapidly migrating cells generally moving longer distances. As neurons often briefly reversed the direction in which they were migrating, or reached the end of a glial fiber and then paused before reversing their direction, the actual distance a given neuron moved over the entire observation period was generally underestimated.

Anti-astrotactin antibodies block neuronal migration in vitro

To examine the role of the neuron-glial ligand astrotactin in neuronal migration, we carried out antibody perturbation experiments. In the presence of the IgG fraction of anti-astrotactin antisera (Stitt and Hatten, 1990), the average speed of neuronal migration along glial fibers was reduced by sixty-one percent

($t=848$ $P<0.05$, Wilcoxon signed rank: representative single trial comparison) to a rate of $4\text{--}5\ \mu\text{m h}^{-1}$ (Fig. 1A and C). Over a five hour observation period, eighty-five percent of the neurons move at rates slower than $8\ \mu\text{m h}^{-1}$; sixty percent were stationary ($0\text{--}4\ \mu\text{m h}^{-1}$), as compared to less than twenty percent of neurons in control cultures (cf. Fig. 1A and B).

The inhibition of migration seen in the presence of anti-astrotactin antibodies was concentration-dependent. The addition of $0.1\text{--}0.2\ \text{mg ml}^{-1}$ of anti-astrotactin antibodies (IgG fraction) reduced glial-guided neuronal migration by twenty percent. At $>1.0\ \text{mg ml}^{-1}$ neuronal migration was reduced by approximately sixty percent. Higher concentrations of anti-astrotactin antibodies ($>2.0\ \text{mg ml}^{-1}$) did not cause further reduction. Similar results were obtained with anti-astrotactin Fab fragments. Maximal inhibition ($\sim 60\%$) occurred at $0.5\text{--}1.0\ \text{mg ml}^{-1}$. In the presence of anti-astrotactin Fab fragments, migrating neurons began to detach from the glial fibers within three hours of the antibody addition to the cultures. By contrast, although anti-astrotactin antibodies blocked glial-guided neuronal migration, the neurons remained bound to radial astroglial fibers throughout the three hour assay period.

Anti-L1, anti-N-CAM, anti-TAG-1, and anti- β_1 integrin antibodies do not block neuronal migration *in vitro*

To examine the contribution of neural cell adhesion molecules of the IgG superfamily and β_1 integrin to glial-guided neuronal migration, migration was assayed in the presence of antibodies (or Fabs) against L1, N-CAM, TAG-1 and β_1 integrin. The addition of these antibodies (or Fabs), either individually or in combi-

nation, did not alter the average rate of migration compared with that observed in control cultures (Fig. 2A).

To determine whether antibodies against N-CAM, L1 or TAG-1 would further reduce the average speed of migration observed in the presence of anti-astrotactin antibodies, we analyzed combinations of antibodies against L1, N-CAM, TAG-1 and astrotactin. No additional inhibition of neuronal migration was seen when combinations of antibodies were analyzed. As a positive control, we previously demonstrated that the anti-astrotactin antibodies blocked neuron–glia adhesion *in vitro*, that the anti-L1 and anti-N-CAM antisera that we assayed blocked neuron–neuron adhesion *in vitro* and that the β_1 integrin (anti GP40) antibody that we assayed detached fibroblasts from a culture substratum (Stitt and Hatten, 1990).

Time course of changes in the cytology of migrating neurons after the addition of anti-astrotactin antibodies

To resolve the effects of anti-astrotactin antibodies on the motility of migrating neurons in real time, we mounted the microcultures in a Berg perfusion chamber, identified a migrating neuron with high magnification AVEC-DIC microscopy, perfused in anti-astrotactin antibodies while the cell was migrating, and examined the cytology and movement of the neuron (Fig. 3). AVEC-DIC microscopic analysis of approximately 50 migrating neurons showed that the mean rate of migration was reduced by sixty percent within 15 min of the addition of anti-astrotactin antibody (c.f. Fig. 1A). The motile activity of the leading process decreased, directional streaming of vesicles from the cytoplasmic into the leading process ceased, and the position of the neuronal nucleus shifted from its

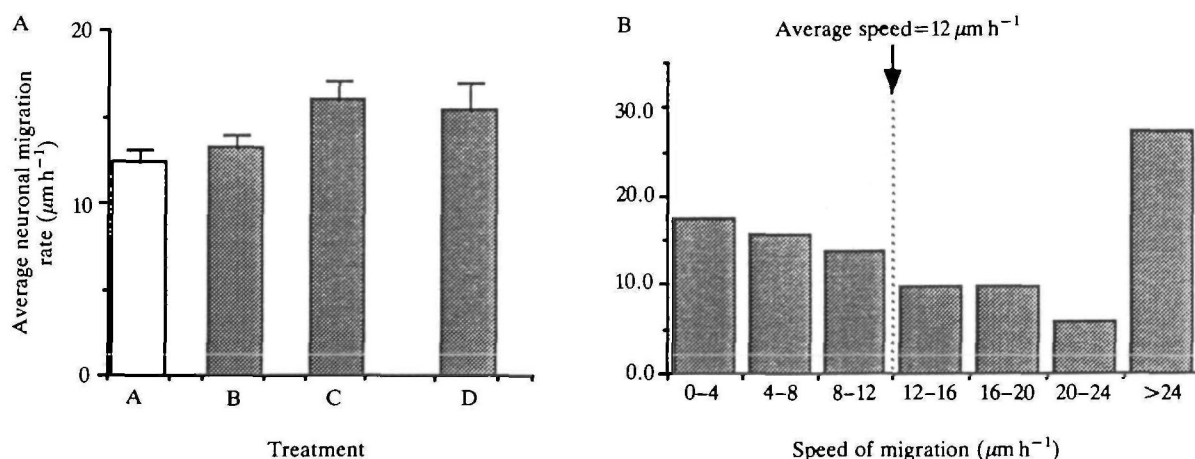


Fig. 2. Antibodies against L1, TAG-1, N-CAM, β_1 integrin or combinations thereof do not change the rate or frequency of granule neuron migration *in vitro*. In A the mean rate of migration of granule neurons in the presence of L1 antibodies (treatment B), N-CAM antibodies (treatment C) (both at antibody concentrations of $1.0\ \text{mg ml}^{-1}$) and a combination of L1, N-CAM and β_1 integrin antibodies (treatment D) (each antibody at a concentration of $0.5\ \text{mg ml}^{-1}$) is compared to the mean rate of migration under control conditions (treatment A). None of these antibody treatments significantly change the rate of granule neuron migration. In B the percent distribution of the migration rates of a population of granule neurons in the presence of a combination of antibodies against L1, N-CAM and β_1 integrin (treatment D) is shown. The distribution of migration rates in the presence of this combination of antibodies is indistinguishable from that observed under control conditions (c.f. Fig. 1A).

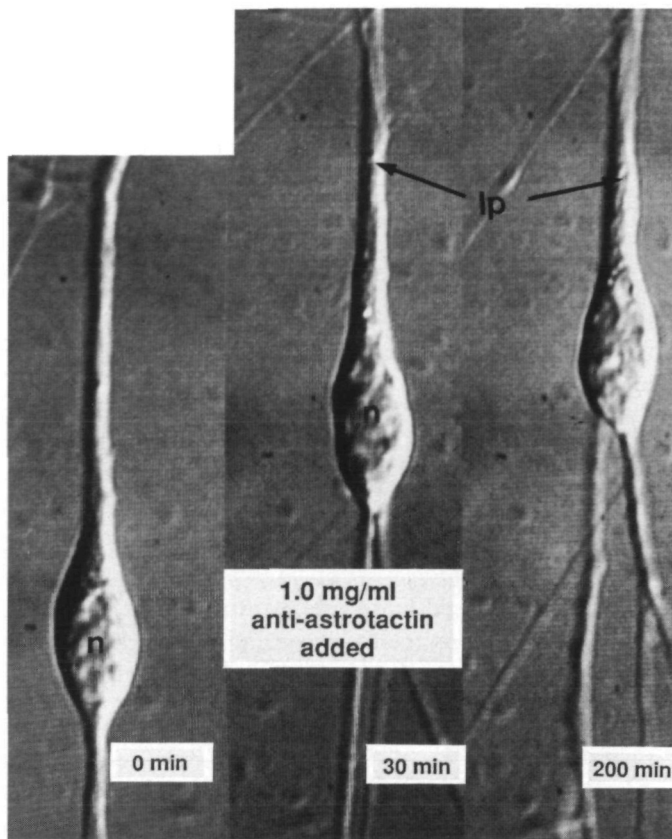


Fig. 3. Anti-astrotactin rapidly arrests neuronal migration *in vitro*. A single migrating granule neuron is shown before, during and after the addition of 1.0 mg ml^{-1} of anti-astrotactin antibodies, using high-power DIC optics ($100\times/1.6$). Prior to the addition of antibodies this neuron displays the cytology and activity of a typically migrating neuron. The addition of anti-astrotactin antibodies (at thirty minutes) rapidly arrested further neuronal migration. Observation of this cell with high resolution video-microscopy also revealed that the directed transport of vesicles into the leading process was lost coincident with the arrest of neuronal migration. Three hours after the addition of the antibodies, the leading process (lp) of the migrating neuron collapsed and started to withdraw. Similarly the neuronal nucleus (n) moved from its typical caudal position, to a more central position in the cell soma. Non-directional neuronal blebbing continued within the neuron subsequent to the anti-astrotactin-mediated block of neuronal migration.

characteristic caudal position to the center of the cell soma. Although these changes in the motility and cytology of the cell were evident over the first hour of treatment of anti-astrotactin antibodies (or Fabs), the neuron remained extended along the glial fiber. The adhesion of both the neural soma and leading process to the glial fiber remained intact. Two to three hours after perfusion of anti-astrotactin antibodies, the leading process collapsed and then retracted over the subsequent 3 h period. As seen in the quantitative phase-contrast analysis, in the presence of anti-astrotactin Fabs, neurons began to dissociate from the astroglial fibers within 3 h.

To analyze the reversibility of the effects of anti-astrotactin antibodies on neural migration, we washed out the antibodies by perfusion with fresh media. When antibodies were removed after 1–3 h, the effects of anti-astrotactin antibodies could be partially reversed. However, in these experiments neurons never regained the migration rate seen in untreated cultures. Even eight to ten hours after anti-astrotactin antibodies were removed, the average rate of migration remained approximately $8 \mu\text{m h}^{-1}$. As a control, we examined the effects of fresh medium and of antibodies against L1 and N-CAM. By contrast to the results with anti-astrotactin antibodies or Fabs, the perfusion of fresh media or antibodies against L1 and N-CAM (or combination of the two) onto actively migrating neurons did not alter the motility or cytology of the cells.

Correlated video and electron microscopy of anti-astrotactin antibody-treated neurons

To examine the effects of anti-astrotactin antibodies on the cytology and neuron–glia apposition of migrating neurons, we carried out electron microscopy of migrating granule cells (Gregory *et al.* 1988). Cell migration was monitored in scribed fields with AVEC-DIC microscopy for 3 h, after which we immediately fixed the cells and processed them for electron microscopy (Fig. 4). As a control, we examined the cytology and neuron–glia apposition of untreated neurons and cells treated with anti-N-CAM antibodies.

The addition of anti-astrotactin antibodies induced dramatic changes in the cytological features of migrating neurons. At the EM level, the most prominent change was the absence of the leading process, giving many cells a club-like appearance rather than the bipolar configuration characteristic of migrating neurons (Gregory *et al.* 1988). Numerous, short filopodia extended from the surface of the neural soma (Fig. 4B). Loss of orientation of filopodial extension was previously shown to be characteristic of stationary, but not migratory granule cells (Gregory *et al.* 1988). Anti-astrotactin antibodies also disrupted the interstitial junction along the neural soma (Fig. 4C). Whereas untreated cells exhibited thickening of the membranes and dilation of the interstitial space along the length of the soma, discrete, *puncta adherentia* junctions were seen along the apposition of the soma and glial fiber in some anti-astrotactin-treated cells.

Previous observations of migrating neurons suggested a network of filaments extending into the leading process, as well as fine cytoskeletal elements which project into the interstitial junction (Gregory *et al.* 1988). Treatment of the cells with anti-astrotactin antibodies resulted in tangles of cytoskeletal elements in the area rostral to the nucleus (Fig. 4A). The mitochondria within neurons, in anti-astrotactin antibody-treated cultures, were distended and their cristae appeared to be irregular. By contrast to the results obtained with anti-astrotactin antibodies, the addition of anti-N-CAM antibodies did not perturb the cytology

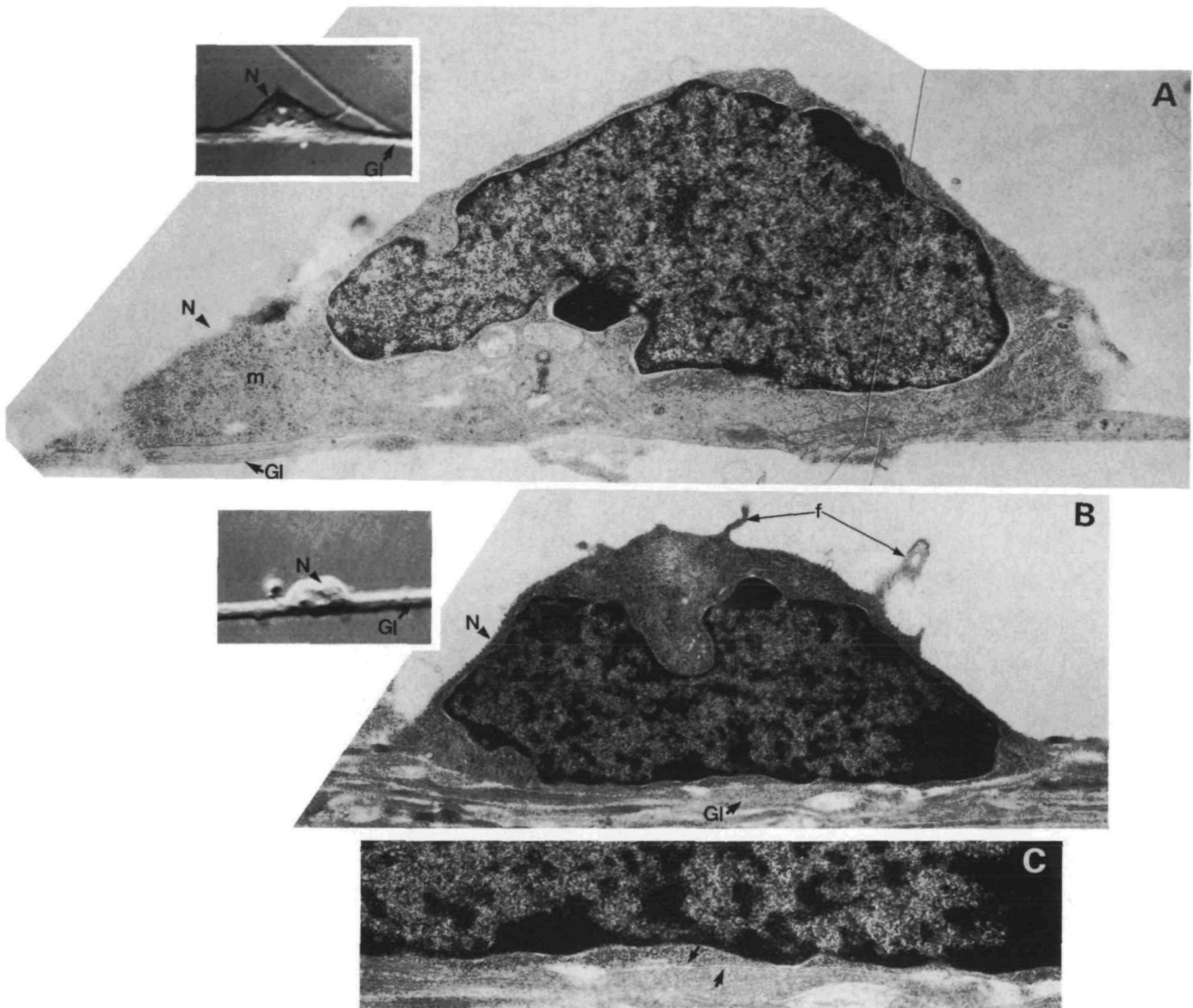


Fig. 4. Correlated video and electron microscopy of granule neurons in the presence of anti-astrotactin antibodies. In A the electron microscopy profile of a granule neuron in the presence of anti-astrotactin antibodies is visualized. The inserted photomicrograph in the upper left hand corner reveals how the same neuron appeared using DIC microscopy. The leading process of this neuron has diminished to a small protuberance extending to the left of the cell nucleus. In contrast to neurons migrating under control conditions, the microtubules (m) within the leading process appear to be disorganized. The junction between the neuron and underlying glial fiber persists. In B a neuron after a more prolonged period of anti-astrotactin antibody exposure is shown. The inserted photomicrograph in the upper left-hand corner reveals how the same neuron appeared using DIC microscopy. All evidence of the polarity seen in migrating neurons is lost. The granule neuron's nucleus is located in the center of the cell soma. Similar to that observed in resting neurons, the surface of the neuron away from the glial fiber displays active filipodial (f) extension. In C a higher power view of the neuron-glial junction seen in B is shown. Rather than the continuous interstitial junction seen along the length of the cell soma of migrating neurons, neuron glial contact has been reduced to *puncta adherentia* junctions along the apposition of the neuronal cell soma and the adjacent glial fiber.

or neuron-glial adhesion of migrating granule neurons (not shown).

Expression of astrotactin, N-CAM, L1 and β 1 integrin on the surface of migrating neurons

To localize the distribution of astrotactin, N-CAM, L1 and β 1 integrin on migrating neurons, cultures of migrating neurons were immunostained with the antibodies used for perturbation assays. Antisera

against L1, N-CAM, TAG-1, β 1 integrin and astrotactin all labeled the cell surface of migrating granule neurons. Confocal imaging of immunolabeling permitted resolution of the pattern of neuronal cell surface labeling. Anti-L1 staining was finely speckled along the surface of both granule neurons and astroglial processes (Fig. 5A). Anti-TAG-1 (not shown) and anti- β 1 integrin, anti-N-CAM and anti-astrotactin all show punctate labelling of the surface of migrating neurons.

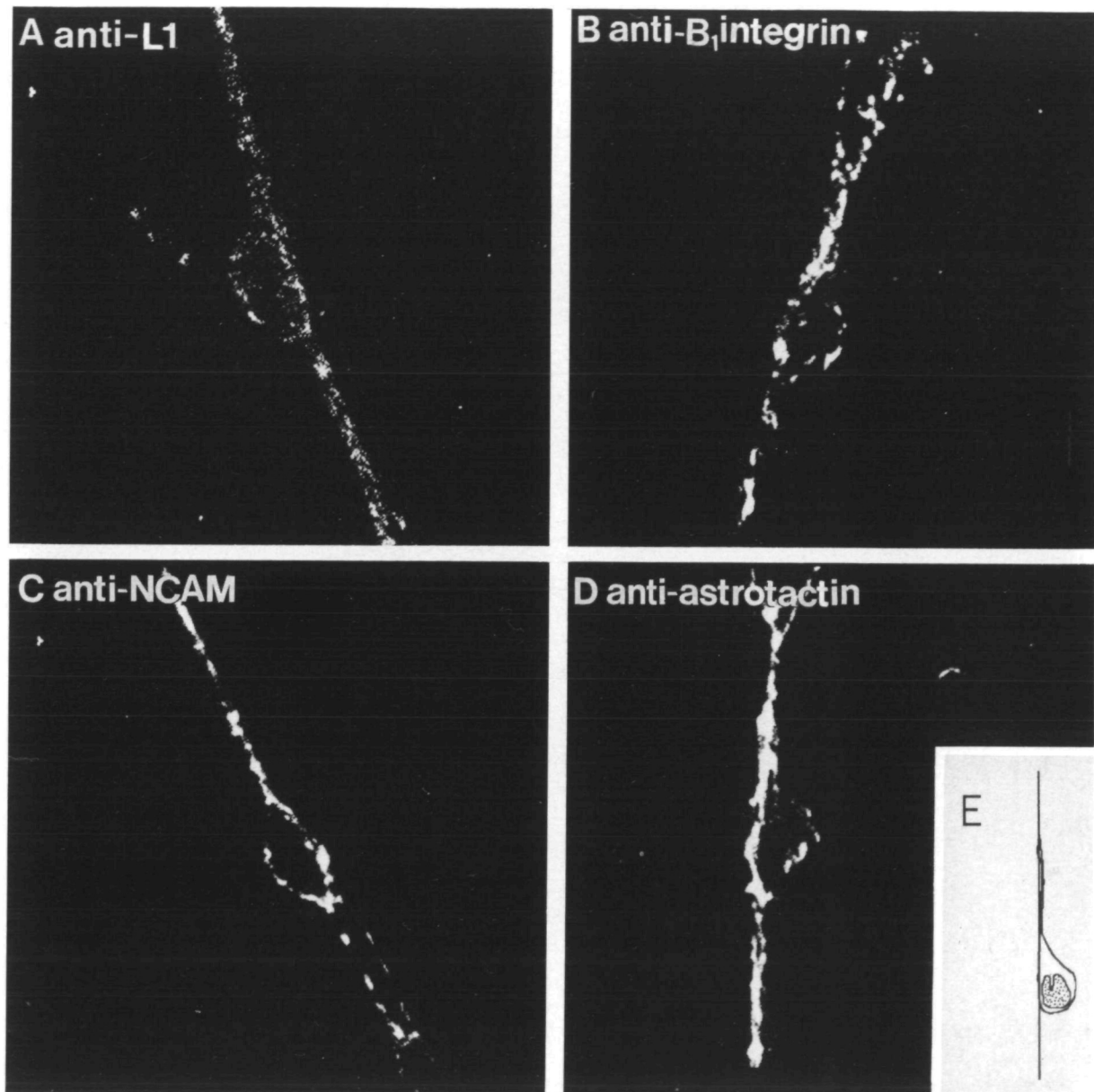


Fig. 5. Immunolocalization of antibodies against, L1, β_1 integrin, N-CAM, and astrotactin on migrating granule neurons *in vitro*. In A–D the immunolocalization of antibodies against L1, β_1 integrin, N-CAM and astrotactin is shown. All antisera label the cell surface and leading process of migrating neurons. (A) Anti-L1 labelling was finely speckled along the surface of both granule neurons and astroglial processes. (B) Anti- β_1 integrin and (C) anti-N-CAM staining exhibit punctate pattern of labeling in the cell body and leading and trailing process of the migrating neurons. (D) Anti-astrotactin gave a staining pattern that was similar to that seen with anti-integrin and anti-N-CAM. Notably extensive immunofluorescence was visualized along the cell soma and interstitial junction. (E) A simplified drawing of the orientation of the migrating neurons shown in A–D.

Notably neurons stained with anti-astrotactin show extensive immunofluorescence along the cell soma and interstitial junction (Fig. 5D).

Discussion

In cortical regions of developing brain, the radial glial fiber system provides the primary pathway for neuronal migrations (Rakic, 1972; Rakic, 1990; Misson *et al.*

1990). The present analysis of granule neuron migration along a glial fiber system *in vitro* demonstrates that neurons move along glial fibers at an average speed of $12 \mu\text{m h}^{-1}$, with individual cells having speeds ranging from $8\text{--}70 \mu\text{m h}^{-1}$. Antibody perturbation assays demonstrate that among the neural cell adhesion molecules of the IgG superfamily, β_1 integrin and astrotactin, the neural antigen astrotactin functions as the primary receptor system in glial-guided neuronal migration. The effects of anti-astrotactin antibodies are rapid, leading

to an arrest of motility and loss of the cytological features of migrating cells.

The observation that 90% of the population of migratory cells showed reduced migration rates in the presence of anti-astrotactin antibodies suggests that astrotactin is utilized by all neurons during glial-guided migration (Fig. 1). In the presence of these antibodies, migrating neurons lost their characteristic features, which include the extension of a motile leading process in the direction of migration and the formation of an interstitial, adhesion junction along the cell soma. Viewed with AVEC-DIC microscopy, the initial effects of the astrotactin antibodies (15 min) were to arrest the extension of the leading process along the glial fiber, suggesting that astrotactin is required for the formation of new sites of adhesion along the leading process (Fig. 3). The slow rate of dissociation of the neural soma from the glial fiber in the presence of anti-astrotactin antibodies suggested that the interstitial junction is a more stable adhesion than the *puncta adherentia* along the leading process.

The conclusion that the anti-astrotactin antibodies released neuron–glia adhesions in the interstitial junction is supported by the present immunocytochemical localization of antibodies against astrotactin, showing that astrotactin is present along the length of the neural cell soma, the site of the interstitial junction. This conclusion is further supported by our previous finding that anti-astrotactin antibodies block the binding of granule cells and of granule cell membranes to astroglial cells *in vitro* (Stitt and Hatten, 1990). The latter studies also established that astrotactin is the neural component of a heterophilic neuron–glia adhesion system, as preincubation of the neuronal membranes, but not of the glial cells, with the anti-astrotactin antibodies inhibited membrane binding. At present, the glial receptor(s) for astrotactin is not known.

The critical role of astrotactin in neuronal migration, suggested by the present antibody perturbation assays, is supported by membrane binding studies showing that astrotactin is the neuronal membrane component of a neuron–glial binding system of early postnatal cerebellar cells (Stitt and Hatten, 1990). It is also supported by our observation that astrotactin is expressed by CNS neurons and not glial cells (Edmondson *et al.* 1988; Stitt *et al.* 1990) and by immunocytochemical localization of the astrotactin antibodies *in vivo*, showing that astrotactin is expressed by migrating neurons and by neurons during periods of assembly into neuronal layers in developing brain (Stitt *et al.* 1990). Western blot analysis indicates that the astrotactin antibodies that block neuron–glia binding in functional assays recognize a band at of approximately $100 \times 10^3 M_r$ in Triton X-100 extracts of early postnatal cerebellar cell membranes (Stitt and Hatten, 1990). The present analysis does not rule out the possibility that minor components present in the antiserum that we assayed contributed to the inhibition of neural migration that we observed.

Antibody perturbation analyses of granule neuron migration *in vitro* did not indicate a critical role for the

immunoglobulin-like adhesion molecules in the movement of the neuron along the glial fiber. This is supported by recent analyses of the receptor systems in granule neuron binding to astroglia (Stitt and Hatten, 1990), showing that N-CAM, L1, or TAG-1 do not contribute to the binding of neuronal membranes to astroglial cells. The conclusions as to the function of N-CAM and L1 in neuronal migration are supported by positive controls showing that the antibodies that we used in migration assays block neuron–neuron adhesion *in vitro* (Stitt and Hatten, 1990). The conclusions regarding TAG-1 (Dodd *et al.* 1988; Furley *et al.* 1990) are less strong, as the anti-TAG-1 antibodies that we assayed have not been demonstrated to block neural adhesion. The finding that antibodies against these neural adhesion molecules did not act synergistically with anti-astrotactin antibodies further supports the conclusion that these receptor systems do not contribute to neuronal migration along glial fibers. These results do not, however, rule out the possibility that other, novel neuron–glia adhesion systems or integrins contribute to neuronal migration, as multiple receptor systems have been shown to contribute to growth cone and fibroblast locomotion (Chang *et al.* 1987; Tomaselli *et al.* 1986, 1988; Neugebauer *et al.* 1988; Elkins *et al.* 1990; Drazba and Lemmon, 1990).

The present results contrast with findings using cerebellar slice assay systems, which have suggested that several neural adhesion molecules, including the neural cell adhesion molecules L1 (Lindner *et al.* 1983) and N-CAM (Lindner *et al.* 1983; Choung *et al.* 1987), cytactin (Chuong *et al.* 1987), and the glial antigen AMOG (Antonicek *et al.* 1987) contribute to neuronal migration. Slice preparations offer the advantage that the complex geometry of cells within cerebellum is maintained and that the preparation can be observed over days to assess the effect of blocking antisera. The limitation of the slice system, however, is the inability to determine the site of action of the antibody. In the case of L1, for example, immunoelectron microscopic localization studies indicate that L1 is expressed by the granule cell neurites, the parallel fibers, rather than at the neuron–glia apposition of the cell soma of migrating cells (Persohn and Schachner, 1987). This, together with studies showing that L1 promotes axon growth along axons (Rathjen and Schachner, 1984), suggests that the effects of antibodies against L1 on granule cell migration are indirect, acting to inhibit parallel fiber fasciculation rather than impede migration of the neural soma along the glial fiber.

The effects of anti-astrotactin antibodies are similar to results obtained with many types of cells after treatment with antibodies against integrins. In these other cell types, $\beta 1$ integrin has been shown to provide a cell attachment domain on the external surface of the cell and a linkage to the cytoskeleton on the cytoplasmic domain of the protein (Damsky *et al.* 1979; Damsky *et al.* 1985; Burridge, 1986; Horwitz *et al.* 1986; Buck and Horwitz, 1987; Chen *et al.* 1985; Hynes, 1990). However, the failure of antibodies against $\beta 1$ integrin to inhibit migration suggests that $\beta 1$ integrin does not

contribute to glial-guided neuronal migration. This result is consistent with previous studies showing that these antibodies do not perturb the binding of granule cell membranes to astroglial cells *in vitro*, and with the finding that calcium-independent adhesion mechanisms account for approximately 85–90% of granule neuron–glia binding (Stitt and Hatten, 1990).

An interesting feature of antibody-arrested migration in living cells was the cessation of organelle and vesicle flow into the leading process. Previous video microscopy (Edmondson and Hatten, 1987) and correlated video and electron microscopy studies (Gregory *et al.* 1988) established the flow of vesicles forward from the cell soma into the leading process as a characteristic feature of migrating neurons. As axoplasmic vesicle flow has been shown to occur along microtubules (Schnapp *et al.* 1985; Vale *et al.* 1985), the disruption of the movement of organelles by the anti-astrotactin antibodies further suggests that astrotactin is interconnected, on the cytoplasmic side, with cytoskeletal elements. One interpretation of our results is that treatment with the antibodies induced release of the cytoskeleton from neuron–glia adhesions which, in turn, disrupted the microtubule-based organelle transport system of the cell. The effect of the anti-astrotactin antibodies on the cytoskeleton of migrating cells supports this idea. By electron microscopy, treatment of migrating cells with the antibodies disorganized the cytoskeleton, tangling cytoskeletal elements within the cytoplasm of migrating cells and disrupted the cytology of microtubule associated mitochondria (Fig. 4). Previous studies, utilizing correlated video and electron microscopy, demonstrated a system of microtubules extending from a basal body forward of the nucleus of migrating cells rostrally into the leading process, as well as a network of membrane-associated, fine, filaments projecting from the cytoplasm of the neuron into the interstitial, neuron–glia junction (Gregory *et al.* 1988). One interpretation of these findings is that the cytoskeletal organization of migrating neurons is provided by specific neuron–glia adhesions, the *puncta adherentia* along the leading process and the interstitial neuron–glia junction along the neural soma.

The present study demonstrates that the neuronal receptor systems utilized in neuronal migration along the glial fiber system differ from those utilized in neural crest cell migration along ECM components and from those utilized in neurite outgrowth on glial substrata. Whereas neural cell adhesion molecules and calcium-dependent adhesion systems, including $\beta 1$ integrin, are prominent in the latter forms of motility (Damsky *et al.* 1985; Chen *et al.* 1985; Bronner-Fraser, 1985; Bronner-Fraser, 1986; Horwitz *et al.* 1986; Buck and Horwitz, 1987; Reichardt *et al.* 1989; Hynes, 1990), these neural receptor systems do not appear to contribute to glial-guided migration. Moreover, the mechanism of movement of CNS neurons along glial fibers appears to involve a different mode of motility, the formation of an interstitial junction, and saltatory contraction and extension of the neural soma. The neural protein astrotactin contributes to the establishment of adhesion

sites along the leading process, the formation of new interstitial junctions and neural cytoskeletal organization, as the neuron moves along the glial guide.

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